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14. ABSTRACT

This project is a Partnering PI option with Dr. Jianguo Cheng at CCF as Initiating PI and Dr. Tingyu Qu at UIC as Partnering PI. The specific aims are to generate functional chromaffin-like cells from mesenchymal stem cells (MSCs) and to investigate the analgesic and anti-tolerance effects and the safety of chromaffin-like cells in animal models. We have conducted the proposed experiments as outlined in SOW. Specifically, we have produced chromaffin-like cells by reprogramming human MSCs (hMSCs) with the extracts of porcine adrenal chromaffin cells. We have harvested bone marrow tissues from rats and isolated, cultured, and expanded rat MSCs (rMSCs) for the targeted reprogramming by using cellular extracts of porcine adrenal chromaffin cells to produce chromaffin-like cells. Recently, we found that even naïve MSCs at their early passages (<passage 5) had significant analgesic and robust anti-tolerance effects in both cellular and animal models. Our research has led to one poster presentation at conference, one patent application, and one manuscript in preparation in the second year of this 3 year project.

15. SUBJECT TERMS
Mesenchymal stem cells, Cell reprogramming, Pain management, Tolerance, Drug abuse, Cell cultures, Spinal transplantation of autologous stem cells, Animal behavioral tests

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INTRODUCTION:

Problems: Pain is a leading cause of disability among active duty and retired military personnel. Ineffective treatment often leads to pain-related impairments and drug abuse with long-term costs to both the military health and disability systems. Clinical trials have demonstrated that transplantation of allogeneic adrenal chromaffin cells provides significant pain relief in patients with intractable cancer pain and in patients experiencing allodynia, a hallmark of neuropathic pain (1, 2, 3, 4). Following adrenal chromaffin cell transplantation, there is often a long-term pain relief without analgesic tolerance (one year in humans) (5), application of exogenous opiates can further alleviate pain without dosage escalation (4); i.e., there was a stabilization of exogenous analgesic intake in these patients, strongly indicating that transplantation of adrenal chromaffin cells ameliorated the problem of opioid tolerance (2, 6). These adrenal chromaffin cells release a "cocktail" of endogenous analgesic substances, including enkephalins, catecholamines, gamma aminobutyric acid, indolalkylamines, and other neuropeptides (7, 8). The analgesic effects of chromaffin cells can be partially reversed by intrathecal injection of either the opioid antagonist naloxone or the adrenergic antagonist phentolamine (9, 10, 11), suggesting that these effects are mediated largely by opioids and catecholamines released by these cells. The anti-tolerance analgesic effects produced by the transplantation of adrenal chromaffin cells may be attributed to a synergistic action of endogenous analgesic molecules released by the transplanted chromaffin cells.

However, clinical practice has been hindered due to the limited availability of suitable human adrenal tissue, genetically well-matched donors in particular, to serve as grafts. Mature chromaffin cells are post-mitotic when they produce enkephalines and catecholamines. Thus, the expansion in culture of these cells is not possible. Xenogeneic materials, such as bovine and porcine chromaffin cells, have been extensively studied as potential alternative materials to human chromaffin cells. Transplantation of these xenogeneic cells into the spinal subarachnoid space produces antinociceptive effects on both Aδ and C fiber-mediated responses in a thermal pain model of rat and non-human primate (12, 13, 14, 15), with a gradual decline in analgesic efficacy that can be prolonged by administration of immunosuppression (13, 15), suggesting that xenogeneic chromaffin cells elicit host immunological rejection to the transplants, and that immunosuppressive therapy is necessary for enhancing long-term graft survival to extend the analgesic effect of the transplants. In addition, there has been concern regarding pathogen contamination of these xenogeneic materials, such as bovine spongiform encephalopathy for bovine chromaffin cells. Thus, the ideal cell source would be the autologous chromaffin cells derived from the patient's own tissue.

Emerging cell reprogramming technology allows production of chromaffin-like cells (CLCs) from autologous stem cells (16, 17, 18, 19), which can be generated epigenetically to secrete analgesic substances, anti-inflammatory factors, and immunological modulating molecules, and used for the management of chronic pain and prevention of drug abuse. In our preliminary studies, we have successfully generated functional chromaffin-like cells by reprogramming human mesenchymal stem cells (hMSCs) with the cellular extracts of porcine chromaffin cells (20, 21, 22). We hypothesized that transplantation of these reprogrammed CLCs, autologous CLCs in particular, will produce significant long term analgesic and anti-tolerance effects without any major adverse effects and immunological rejection. This project is a Partnering PI option with Dr. Jianguo Cheng at CCF as Initiating PI and Dr. Tingyu Qu at UIC as Partnering PI. They will work jointly on the Specific Aims to generate CLCs from hMSCs and rat MSCs (rMSCs) and to investigate the analgesic and anti-tolerance effects and the safety of CLCs in comparison with naïve MSCs and porcine chromaffin cells in cell cultures and animal pain models. The success of this project is anticipated to establish an innovative therapy that will have a profound impact on pain management and drug abuse prevention, two of the major barriers facing the military and society. Such a therapy will fundamentally reduce the need for exogenous opioid medications and minimize the risk of prescription drug abuse and addiction. It can be used in hundreds of thousands of patients with a wide range of cancer and non-cancer pain states to improve quality of life and save billions of dollars for the military health and disability systems.

BODY:

This partnering PI option project has been progressing as planned. We here report our research progress and accomplishments in the second 12 months of this 36 month project as outlined in our original statement of work (SOW) for the partnering PI's specific tasks in the Qu lab in details including methodology, results, and discussion of data.

Task 1 (month 1-32): Generate functional chromaffin-like cells: i.e. to use porcine chromaffin cell nuclear and cytoplasmic extracts to reprogram hMSCs and rMSCs for producing analgesic chromaffin-like cells (CLCs). Task 1 is in progress and continues to produce CLCs for *in vitro* and *in vivo* experiments as stated in SOW until the completion of this project.

We have produced human CLCs (hCLCs) by reprogramming hMSCs with the extracts of porcine adrenal chromaffin cells (24) based on our established protocol (20, 21, 22). The experiments for the production of rat CLCs (rCLCs) from rMSCs is on-going in the lab. As controls, non-reprogrammed hMSCs and rMSCs at early stage (<passage 5) and late stage (>passage 10) as well as the porcine chromaffin cells were isolated, cultured, or expanded. These cells are shipped to Dr. Cheng's lab periodically according to experimental needs, as we performed in last year, for *in vivo* cell transplantation studies in rat pain models.

1a. Acquisition of porcine chromaffin cells for CLC production:

In this year, we purchased twenty porcine adrenal glands from Sierra For Medical Science (Whittier, CA), and all of the porcine adrenal glands (x20) were processed for chromaffin cell isolation. The protocol used to isolate porcine adrenal chromaffin cells from these adrenal glands is the same as we employed previously (20, 21, 22, 24). In brief, adrenal glands were manually perfused three times with warmed (37°C) Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM NaHCO3, 5.6 mM glucose, 5 mM HEPES (Sigma, St. Louis, MO), pH 7.4, supplemented with 100 UI/ml penicillin, 100 mg/ml streptomycin, and 0.125 ml/ml fungizone (PSF; Sigma, St. Louis, MO), followed by digestion of connective tissue with 0.125% collagenase A (Boehringer Mannheim, Mannheim, Germany) in Locke's buffer for 3 × 5 min at 37°C. At the end of the digestion period, the chromaffin cells were isolated from the dissected medulla by mechanical dissociation. The harvested cells were filtered through a 70-μm nylon mesh (BD Falcon, Bedford, MA, USA) and then centrifuged at 150 × *g* for 10 min in Locke's buffer. The resulting cells were purified on 39.47% Percoll gradients (Pharmacia Biotech, Uppsala, Sweden) by centrifugation at 22,500 × *g* for 20 min. The portion of the gradients containing purified chromaffin cells was harvested by aspiration and washed three times by centrifugation at 150 × *g* in Locke's buffer. Freshly isolated chromaffin cells were suspended and plated in 75 cm^2 culture flasks (Corning, Cambridge, MA) containing Dulbecco's modified eagle medium/F12 (DMEM/F12, 1:1; Gibco, Grand Island, NY) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma, St Louis, MO) and antibiotics (PSF). These chromaffin cells were maintained at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA) fed by replacing culture media twice per week and used for experiments within four weeks after culturing because mature chromaffin cells are post-mitotic and do not survive longer in cultured condition.

1b. Cell reprogramming:

Task 1b is in progress.

hMSC culture: hMSCs were purchased from Cambrex (Walkersville, MD) and AllCells (Emeryville, CA). These cellsare negative for surface markers associated with hematopoietic cells [e.g., cluster of

differentiation 11b (CD11b), CD33, CD34, and CD133 antigens] and used for the designed experiments. hMSCs were cultured and expanded using the protocol previously developed in our laboratory (20, 21, 22, 24). In brief, hMSCs were plated in 75 cm^2 culture flasks (Corning, Cambridge, MA) at a concentration of $1x10^5$ cells/cm^2 and cultured in 20 ml growth medium consisting of DMEM (Gibco, Grand Island, NY), an antibiotic-antimycotic mixture (1:100, Invitrogen, Carlsbad, CA), FBS (Stem Cell Technologies, Vancouver, BC, Canada), and incubated at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA). Cells were passaged by incubating with 0.05% trypsin–EDTA (Gibco) for 5 min at room temperature to gently release the cells from the surface of the culture flask after reaching about 80% confluency. Culture medium was added to stop trypsinization, cells were centrifuged at 350 × *g* for 5 min at room temperature, re-suspended, and transferred into new culture flasks at a concentration of 1 × 105 cells/ cm2 for continuous culture and expansion to reach a sufficient number of cells for experimental uses. hMSCs that underwent less than 10 passages were used for the experiments.

rMSC culture: Totally, fourteen adult rat bone marrow tissues (Sprague-Dawley, SD, male) were obtained from Dr. Cheng's lab, and all of the rat bone marrow tissues (x14) were successfully processed for rMSC isolation. In brief, mononuclear bone marrow cells were isolated and incubated at 37°C in a 5% CO2 humidified incubator. Homogeneous rMSCs were received based on their adherence to plastic in culture and expanded in culture. The conditions for culturing and expanding rMSCs as well as cell passaging were similar to those used for hMSCs. Subpopulations of cultured rMSCs at early stage (≤passage 5) and late stage (>passage 5) were collected, respectively, and shipped to Dr. Cheng (Initiating PI) for cell transplantation experiments.

Reprogramming MSCs with the extracts of porcine chromaffin cells: The cell reprogramming processes for producing hCLCs and rCLCs are similar.

a. Preparation of porcine chromaffin cell extracts: Cultured porcine chromaffin cells were counted and washed in PBS and in cell lysis buffer (20mM HEPES, pH 8.2, 50 mMNaCl, 5mM $MgCl_2$, 1mM dithiothreitol, and protease inhibitors) (Sigma, St Louis, MO), sedimented at 400×*g*, resuspended in 1 volume of cell lysis buffer, and incubated for 30 min on ice. Cell samples were then sonicated in 200µl aliquot on ice with a pulse sonicator (PowerGen 125, Fisher Scientific) in short pulses until all cells and nuclei were lysed, and confirmed by microscopic observation. The lysate was centrifuged at 15,000×*g* for 15 min at 4°C. The supernatant was aliquoted and stored in liquid nitrogen for later use.

b. Permeabilization of MSCs with SLO: MSCs were suspended, washed in Ca^{2+}- and Mg^{2+}-free PBS, and centrifuged at 120×*g* for 5 min at 4°C. The collected MSCs were resuspended in aliquots of $1x10^5$ MSCs/100µl of Ca^{2+}- and Mg^{2+}-free PBS in 1.5 ml tubes. Cell samples were permeabilized with streptolysin O (SLO; Sigma-Aldrich, St Louis, MO) at a final concentration of 200ng/ml and incubated in an H_2O bath at 37°C for 50 min with occasional agitation. The cell samples were then placed on ice, diluted with 200µl cold PBS, and sedimented at 150×*g* for 5 min at 4°C.

c. Reprogramming MSCs with the extracts of porcine chromaffin cells: The permeabilized MSCs ($1x10^5$) were resuspended in 100µl of the extracts of porcine chromaffin cells in a 1.5ml tube containing an ATP regenerating system (1mM ATP, 100µM GTP, and 1mM of each NTP, 10mM creatine phosphate, 25µg/ml creatine kinase) (Sigma, St Louis, MO). Cell samples were incubated in an H_2O bath at 37°C for 1 hr with occasional agitation. To reseal the membranes of MSCs, 1ml of DMEM containing 2mM $CaCl_2$ and antibiotics were added to the tube and incubated at 37°C for an additional 1 hr. Finally, $CaCl_2$-containing DMEM was replaced by fresh DMEM with 10% FBS and reprogrammed MSCs were transferred to culture flasks at a concentration of $1x10^5$ cells/cm^2 and expanded continuously for subsequent experiments.

1c. Characterization of CLCs:

a. hCLCs:

Task 1c for hCLCs is completed as reported in last year. A manuscript entitled "Targeted cell reprogramming produces analgesic chromaffin-like cells from human bone marrow-derived mesenchymal stem cells" has been published in the journal of Cell Transplantation (2013, PMID: 23394594) and attached (ref: 24).

As expected, the hCLCs generated from the reprogrammed hMSCs showed a significantly enhanced expression profile for human pre-proenkephalin (hPPE) gene, a precursor for enkephalin opioid peptides, compared to that of naïve hMSCs. Immunocytochemical examination showed that most of the resultant hCLCs (≥90%) expressed a strong immunoreactivity for methionine enkephalin (Met-enkephalin) and tyrosine hydroxylase (TH), specific cell markers for adrenal chromaffin cells. BrdU-positive staining was detected in a subpopulation of resultant hCLCs, suggesting that some hCLCs may have retained a similar proliferative capability as that of hMSCs while receiving new characteristics of analgesic chromaffin cells, which may make these cells even more valuable because generation of dividing cells that are expandable in culture would provide a sufficient quantity of such cells for targeted use (24).

b. rCLCs:

Task 1c for rCLCs is in progress.

The expression of key genes (RT-PCR) and molecular markers of adrenal chromaffin cells (immunocytochemistry), cell proliferation (BrdU immunocytochemistry), cell viability (trypan blue), cell karyotypes (Giemsa staining), and cell apoptosis (TUNEL) will be performed in rCLCs and are on-going experiments conducted in the lab with the same methods as employed to characterize hCLCs.

1d. The phenotypic stability and secretory function CLCs:

a. hCLCs:

Task 1d for hCLCs is completed, details was reported last year, and the results were published (Cell Transplant, 2013, PMID: 23394594) (24).

The hCLCs generated from the reprogrammed hMSCs remained stable phenotype and expanded in culture with an average cell doubling time of about 72 hours up to one month, undergoing about 8 passages. We did not maintain the culture of the cells post the one month time point after cell reprogramming. The level of Met-enkephalin released by hCLCs was significantly augmented compared to that released by naïve hMSCs for the same number of cells in serum-free cultures (24).

b. rCLCs:

Task 1d for rCLCs is in progress. The experiments on phenotypic stability (cell counting and immunocytochemistry) and secretory function (immunoblot and/or ELISA) for rCLCs have been initiated by using the same methods as employed for hCLCs.

Thus, we have successfully generated hCLCs by reprogramming hMSCs with the cellular extracts of porcine chromaffin cells. More importantly, intrathecal injection of these chromaffin-like cells in rats produced significant antinociceptive effect *in vivo* for responses to both Aδ and C thermal stimuli without

using immunosuppressants (Fig. A, $p<0.01$). Our results suggest that analgesic CLCs can be produced from an individual's own tissue-derived stem cells by targeted cell reprogramming for the application of

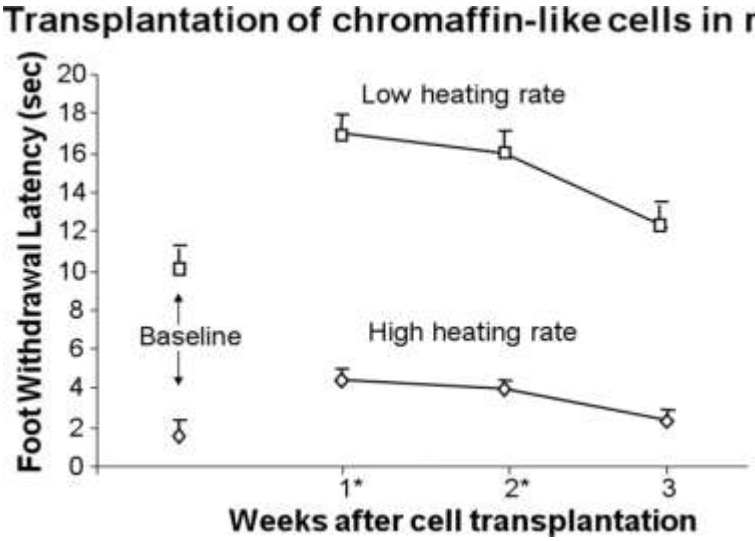

Fig. A. Effects of intrathecal transplantation of a single dose $1x10^5$) of chromaffin-like cells on foot withdrawal latencies evoked by low (C nociceptor: 0.9°C/s, squares) and high (Ad nociceptor: 6.5°C/s, diamonds) heating rates on the dorsal surface of the feet in rats (n = 6). Foot withdrawal latencies were measured before cell transplantation (baseline) and remeasured 1 week and then weekly at 1-week intervals following cell transplantation. Data from baseline and response latencies at different time points after cell transplantation were expressed as the mean ± SEM and compared using t tests. Follow-up analysis was performed by Bonferroni post hoc tests. Results showed that transplantation of chromaffin-like cells increased foot withdrawal latencies evoked by both high and low heating rates for at least 3 weeks, with significant increases compared to baseline for 2 weeks (*$p < 0.01$, t tests and Bonferroni correction).

chronic pain management (24). Transplantation of cells derived from individuals' autologous tissue into the same individuals would be safe and immunocompatible compared to xeno- and allo-transplants. In practice, hMSCs can be derived from a patient's own tissues, easily cultured and expanded, and could not only be prepared to become isogeneic through this cell reprogramming approach, but also could avoid immunological rejection through autologous cell transplantation. Thus, autologous hCLCs could be the most desirable alternative to real adrenal chromaffin cells for potential therapeutic purposes in clinics. Robust and long-lasting analgesic effects are expected for autologous hCLCs because these cells would be spared from immune responses, thereby improving the therapeutic efficacy of the transplanted cells.

Task 2 (month 4-36): Determine the analgesic and anti-tolerance effects and the safety of chromaffin-like cells in rats:

Task 2 is in progress.

Naïve rMSCs, hMSCs, and the generated hCLCs have been shipped to Dr. Cheng's lab, where transplantation studies were conducted to investigate the analgesic and anti-tolerance effects of these cells in a rat model of neuopathic pain. Very promising positive data were produced with a generation of a manuscript (Transplantation of stem cells to treat opioid tolerance). Detailed experimental results will be reported separately by Dr. Cheng (Initiating PI).

2a. Generate neuropathic pain model and perform transplantation experiments; Perform analgesic evaluation; CSF sampling and measuring concentrations of enkephalins and catecholamines.

Task 2a is in progress and is performed in Dr. Cheng's lab. The accomplishment for Task 2a will be reported separately by Dr. Cheng (initiating PI).

2b. Determine cell dose response curve.

Task 2b is in progress and will be reported by Dr. Cheng (initiating PI).

2c. Determine anti-tolerance effects of autologous CLCs to repeated morphine administrations.

Task 2c is in progress.

Previous studies demonstrated that transplantation of adrenal chromaffin cells in humans play an important role in the analgesia and the inhibition of opioid tolerance, which may be attributed to a synergistic action of endogenous molecules released by these cells (1-8). MSCs and/or MSCs-derived CLCs may also produce such analgesic and anti-tolerance effects to chronic opioids. In order to investigate the mechanism(s) of anti-tolerance effects of the cellular therapies, we investigated the potential effect of MSCs to the development of morphine-induced tolerance *in vitro* by a co-culture system of MSCs and the neuronally-differentiated SH-SY5Y cells and *in vivo* by spinal transplantation of MSCs in rat pain models.

Co-cultures of SH-SY5Y cells and hMSCs or HDFs:

SH-SY5Y cells, a well-established stable human neuroblastoma cell line, were purchased from American Type Culture Collection (ATCC, Manassas, VA) and grown in the medium containing Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum, 100μU/ml penicillin, and 100μg/ml streptomycin at 37°C in a 5% CO_2 humidified incubator (Fisher). Before experiments, SH-SY5Y cells were treated with 10μM retinoic acid (RA) for 6 days to induce neuronal differentiation. These differentiated SH-SY5Y cells were then treated with morphine (10μM) for 24 h unless otherwise specified. Human bone marrow samples (n=4, three males, ages 22, 25, and 32; one female, age 50) were purchased from AllCells LLC (Emeryville, CA). The methodogy for isolating and culturing BM-derived hMSCs is the same as we previously described (20-24). In our designed experiments, hMSCs ≤passage 5 were defined as early passage, while hMSCs >passage 10 were defined as the late passage. Co-cultures of SH-SY5Y cells and hMSCs were performed by using the cell culture insert with 0.4μm diameter pores according to the manufacturer's protocol (Greiner Bio-One, Monroe, NC). Briefly, 100μl cell suspension containing different concentrations of hMSCs was pipetted onto the membrane of the cell culture insert (Fig. 1a) or the membrane of the inverted cell culture insert (Fig1. b) and the cells were allowed to adhere overnight at 37°C in a 5% CO_2 incubator. Subsequently, the cell culture insert was placed into the well of a 12 well plate pre-seeded with differentiated SH-SY5Y cells, which were pretreated with 10μM morphine for 24h. Co-cultures of SH-SY5Y cells and hMSCs were maintained for additional 36 hours in the presence of 10μM morphine at 37°C in a 5% CO_2 incubator. The primary human dermal fibroblasts (HDFs) were purchased from Cell Applications, Inc. (San Diego, CA) and cultured in 1640 medium containing 10% FBS, 1% L-glutamine, 1% penicillin/streptomycin at 37°C in a humidified atmosphere of 5% CO_2. The co-cultures of SH-SY5Y

Fig. 1. Diagram of co-culture of MSCs or HDFs/SH-SY5Y cells to show (a) without cell contact, (b) with cell-cell contact.

cells and HDFs of early passages (P≤5) were performed by using the same method described for co-cultures of SH-SY5Y cells and hMSCs at identical condition, as shown in Fig. 1a & b.

cAMP accumulation assay:

cAMP levels, which is involved in opioid tolerance development as one of the cellular mechanisms, was determined in the morphine-treated SH-SY5Y cells. Thirty-six hours after co-cultured with hMSCs or HDFs, SH-SY5Y cells were harvested with a non-enzymatic cell dissociation solution followed by washing once with HBSS buffer. cAMP accumulation in SH-SY5Y cells was assayed by using a LANCE™ cAMP kit (PerkinElmer, Waltham, MA) according to the manufacturer's protocol. SH-SY5Y cells were centrifuged at 1000g for 2 min and re-suspended at a concentration of 2×10^6 cells/ml in stimulation buffer (1×HBSS, 5 mM HEPES, 0.1% BSA, 0.5 mM IBMX, pH 7.4) and mixed with 50μM forskolin. The Alexa pluor®647 labeled antibody was added to the cell suspension and the cells were incubated at 37 °C for additional 15 min, the detection mix was then added to the final cell suspension. The cell sample was further incubated for 1h in the dark and read on a TECAN instrument (San Jose, CA) to measure the LANCE signal. The LANCE signal obtained at 665 nM was directly used to analyze the cAMP levels. The signal at 615nM was used to identify dispensing or quenching problems. The cAMP standard curve was assayed according to the manufacturer's instructions. The cAMP formation was calculated as the percentage of forskolin-stimulated cAMP accumulation without morphine, which was defined as 100% in our experiments. All data were expressed as Mean±SD. Statistical comparisons were analyzed using Prism software. Values of $p<0.05$ were considered statistically significant.

Immuocytochemistry:

Fluorescence immunocytochemical staining was performed on cultured neuronal cells differentiated from SH-SY5Y cells seeded on coverslips. SH-SY5Y cells without co-culture served as controls. Cells were washed with PBS and fixed in 4% paraformaldehyde (Sigma) in PBS (pH 7.4) for 20 min at room temperature. Following washing in PBS, cell samples were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma-Aldrich) and 3% donkey serum (Jackson ImmunoResearch) for 30 min, followed by incubation with rabbit anti-opioid receptor (MOR), (1:1000, ImmunoStar, Inc, Hudson, WI) and mouse anti-Rab5, a marker of early endosomes, (1:1000, Abcam Inc., Cambridge, MA) antibodies overnight at 4°C. Then the cells were washed in PBS and incubated with corresponding secondary antibodies, including rhodamine (TRITC)-conjugated or fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit IgG (1:200; Jackson ImmunoResearch) and donkey anti-mouse IgG antibodies for 2 h at room temperature in the dark. Finally, the cells were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA, USA), and viewed fluorescence microscopy (Zeiss, Jena, Germany). In total, three independent experiments were performed, and the cells double-immunostained by both antibodies were recorded.

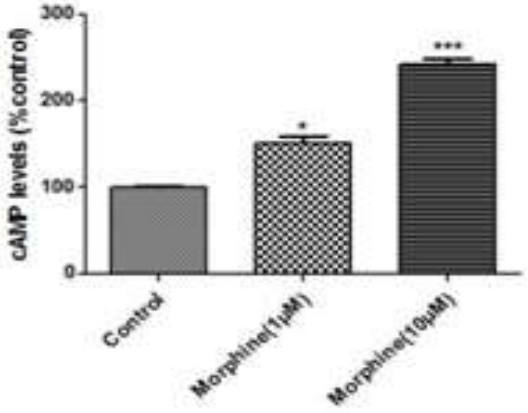

Figure 2: The effect of morphine on cAMP levels in SH-SY5Y cells: SH-SY5Y cells were pretreated with morphine for 24h, and the cAMP levels in SH-SY5Y cells were measured. The cell samples without morphine treatment served as a controls. * p<0.05, *** p<0.001. Data were presented as the Mean ± SEM of three independent experiments.

Results:1.

MSCs inhibited the development of morphine-induced tolerance in SH-SY5Y cell model.

Exposure of 1 and 10μM morphine for 24h led to significantly upregulated cAMP levels in cultured SH-SY5Y cells when compared to the group without morphine treatment (Fig.2, $p<0.05$~$p<0.001$). The cAMP levels were increased to 150.7±20.6 by 1μM morphine and 241.6±19.6 by 10μM morphine, respectively, as shown in Figure 2. The cAMP levels of SH-SY5Y cells were then tested at different time points (0~48h) when treated by 10μM morphine, as shown in Figure 3, Treatment of SH-SY5Y cells with morphine caused significant increase of cAMP concentration in SH-SY5Y cells at 24 and 48h (Fig. 3, $p<0.001$). The levels of cAMP upregulated in the treated SH-SY5Y cells by morphine resulted in ~2.5 fold increase compared to that of SH-SY5Y cells without morphine treatment (Fig. 3). These results are consistent with the previous study (27) and show that SH-SY5Y cells are an appropriate cellular model for investigating the tolerance induced by chronic morphine treatment. Fig. 4 showed that following pretreatment with 10μM morphine for 24h, SH-SY5Y cells were co-cultured with MSCs or HDFs for additional 36h. Results revealed that the upregulation of cAMP

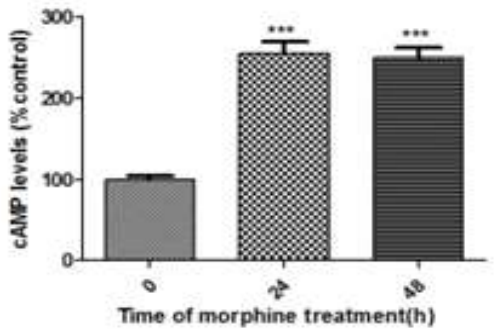

Figure 3: Time course of the development of cAMP upregulation in SH-SY5Y cells: SH-SY5Y cells were incubated with the medium containing 10μM morphine for 48h, cAMP levels were measured at different time points. The cell samples without morphine treatment served as a controls. *** p<0.001. Data represent Mean ± SEM of three independent experiments.

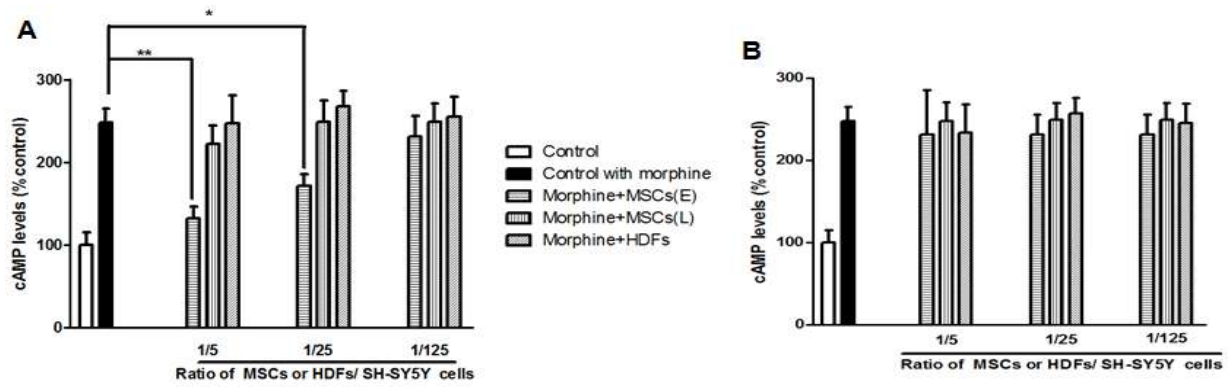

Figure 4: Following pretreated with 10μM morphine, SH-SY5Y cells were co-cultured with MSCs or HDFs with cell-cell contact (a) and without cell contact (b) for 36h at various ratio (1:5, 1:25, 1:125) in a high glucose DMEM medium containing 3% FBS and 10μM morphine and the cAMP levels of SH-SY5Y cells were then assayed. SH-SY5Y cells cultured alone with and without morphine treatment served as controls. Data were presented as the Mean ± SEM of three independent experiments. * p<0.05, ** p<0.01.

induced by morphine in SH-SY5Y cells was significantly attenuated by hMSCs of early passage (≤P5), with $p<0.05$ at a ratio of 1/25 for hMSCs/SH-SY5Y cells and $p<0.01$ at a ratio of 1/5 for hMSCs/SH-SY5Y cells by cell-to-cell contact in the co-cultures, demonstrating a tendency of enhanced inhibition to the development of morphine tolerance in SH-SY5Y cells with the increasing ratio of hMSCs in co-cultures (Fig. 4A). HDFs (≤P5) and hMSCs of late passages (P>10) did not show any detectable inhibitory effect to the morphine-induced cAMP upregulation (Fig. 4A). HDFs are widely considered to be terminally differentiated and lack differentiation and colony-forming potential (34). Our results suggest that the anti-tolerance effect is specific to hMSCs and may be attributed to the interaction of cell-to-cell contact in the co-cultures between SH-SY5Y cells and hMSCs since no such effect was detected in the co-cultures of the same cells without cell physical contact (Fig. 4B). In addition, the proliferation rate of hMSCs of early passages, one of the characteristics of stem cells, is significantly higher than that of

hMSCs of late passages (P>10, data not shown), and hMSCs of late passages may have differentiated or gradually lost their "stemness" during long period of *in vitro* culture. suggesting that the "stemness" of hMSCs at the early stage may also play a crucial role in the anti-tolerance effect observed in our studies. Thus, further studies are warranted to clarify the mechanism(s) of anti-tolerance effect of hMSCs.

Opioid therapy is the cornerstone of pain management (28, 29, 30). However, the long-term use of opioids for the pain management is hampered by analgesic tolerance, which requires escalating doses of drug to maintain pain relief at the same level. Other negative health consequences related with the long term use of opioids include cognitive impairment, drug abuse, and addiction. Although the molecular mechanisms of opioid tolerance are still unclear, the most observed correlative biochemical adaptation both *in vitro* and *in vivo* is the upregulation of cyclic adenosine monophosphate (cAMP) system, including increased intracellular cAMP levels, adenylate cyclase supersensitization, and other chronic changes that involve activation of transcription factors leading to alterations in protein expression (31, 32, 33). Thus, cAMP upregulation after chronic opioids has been proposed as a biochemical mediator underlying chronic effects of opioids and a cellular hallmark to study morphine tolerance. In our experiments to generate analgesic and anti-tolerant CLCs from hMSCs, we found that naïve hMSCs at the early passages (≤passage 5) showed significant inhibitory effect on the development of morphine-induced tolerance in differentiated SH-SY5Y cells, i.e., significantly attenuated morphine-induced cAMP up-regulation (Fig. 4A), suggesting hMSCs may have therapeutic potential for the opioid tolerance treatment in clinics.

2. MSCs increased the expression and co-localization of MOR and Rab 5 in morphine-treated SH-SY5Y-cells.

Immunocytochemical examination of morphine-treated SH-SY5Y cells showed a re-distribuation of mu opioid receptor (MOR) and augmented expression of Rab5, a marker of early endosomes, as well as increased co-localization of MOR and Rab5 when co-cultured with hMSCs of early passages (≤passage 5) (Fig. 5). An important mechanism of opioid receptor regulation involves endocytosis of receptors, failure

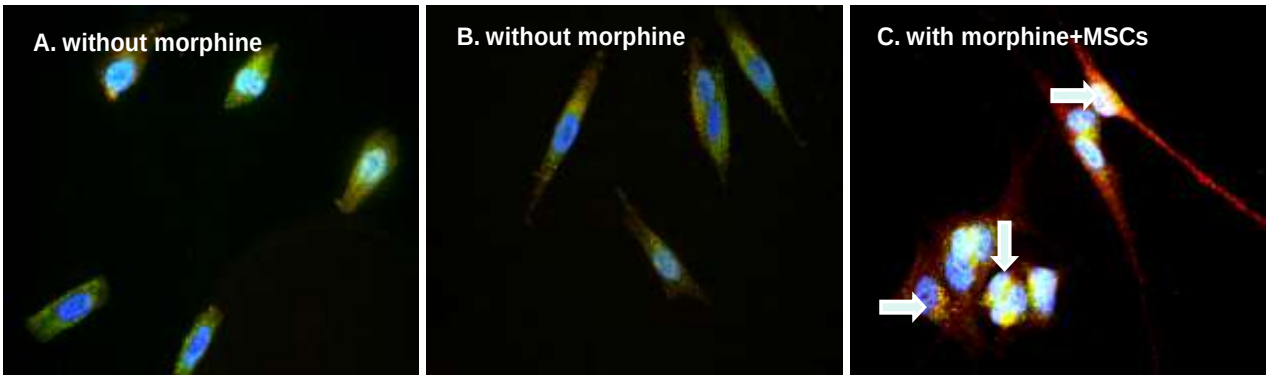

Figure 5. Representative immunocytochemical staining pictures of MOR and Rab 5 in morphine-treatedSH-SY5Y cells processed for fluorescence microscope. In SH-SY5Y cells without (A) and with (B) morphine, MOR (green) and Rab 5 (red) were distributed in the cytoplasm with less Rab 5 immuno staining. In morphine-treated SH-SY5Y cells with hMSC co-culture (C), MOR expression was re-distributed toward the intracellular compartment close to the nuclei with increased co-localization with strong Rab 5 immunostaining (yellow, arrows indicated), suggesting an enhanced activities of MOR and Rab 5 in these cells.

of MOR endocytosis contributes to the development of tolerance and dependence of opioids (35, 36). In SH-SY5Y cellular model, we have shown that hMSCs can inhibit cAMP upregulation induced by chronic morphine, a hall mark of tolerance and dependence, the interesting immunocytochemical results of our studies, i.e., the enhanced activities of MOR and Rab5 of SH-SY5Y cells by hMSC co-culture may be also involved in the reduction of morphine tolerance development in these cells. Thus, we propose the

notion that application of hMSCs may promote the activities of MOR and Rab of neuronal cells to opioids, thereby preventing its tolerance development (23). These findings were presented at the 2nd International Conference and Exhibition on Addict ion Research & Therapy, Las Vegas, USA, July 22-24, 2013 (Abstract is attached). Support of DOD grant is acknowledged in the presentation.

3. MSCs produced significant analgesic and robust anti-tolerance effects in rat pain models:

In the collaboration studies with Dr. Cheng (Initiating PI), the analgesic and anti-tolerance effects of rMSCs at the early stage (passages 2-3) are confirmed in animal pain models by spinal cell transplantation. The details for animal experiments and significance are reported separately by Dr. Cheng.

2d. (months 6-36): Determine the safety of chromaffin-like cell therapy: Determine the fate of the transplanted cells and their phenotypic stability:

Task 2d is in progress.

Based on the current data, the animals with hCLC, hMSC, and rMSC transplantation maintained normal food and water intake, locomotor function, and body weight, no remarkable adverse effect was observed, suggesting the safety of these cells as transplants. Experiments for the fate and phenotypic stability of the transplanted cells are on-going in the lab. In this year, eight postmortem spinal cord samples in total with rMSC (4) and vehicle (4) injections have been received from Dr. Cheng's lab and immunohistological examination was conducted to locate the transplanted cells and determine the cell fate and the host responses to the transplants.

Immunohistochemistry: The lumber segment of the spinal cords covering the site of cell injection was post-fixed overnight and then transferred to a 30% sucrose buffer solution. Sagittal sections (5-20 μm) were cut on a cryostat. Fluorescence immunocytochemical staining was performed on these sections. Following washing in PBS, sections were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma-Aldrich) and 3% donkey serum (Jackson ImmunoResearch) for 30 min, followed by incubation with antibodies overnight at 4°C, including: mouse anti-TH (1:300; Sigma-Aldrich), rabbit anti-Met-enkephalin (1:600; ImmunoStar Inc., Hudson, WI), specific markers for chromaffin cells, rabbit anti-MOR, (1:1000, ImmunoStar, Inc, Hudson, WI), mouse anti-Rab5 (1:1000, Abcam Inc., Cambridge, MA), mouse anti-fibronectin (1:2000, Sigma), a marker for MSCs, and mouse anti CD11b (1:600, Abcam Inc., Cambridge, MA), a marker for microglial cells. Sections were then washed in PBS and incubated with corresponding secondary antibodies, including fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG, and rhodamine (TRITC)-conjugated donkey anti-rabbit IgG antibodies (1:200; Jackson ImmunoResearch) for 2 h at room temperature in the dark. Finally, the sectionss were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA), and viewed with immunofluorescence microscopy (Zeiss, Jena, Germany). No fibronectin stained MSCs were detected in the examined spinal sections (data not shown; suggesting that cells injected into the intrathecal or subarachnoid space of spinal cord may have floated into the cerebrospinal fluid (CSF) from the injection site. The experiments of immuno staining for other cell markers are ongoing in the lab.

Task 3 (months 25-36): Determine the safety of CLCs in SCID mice:

Task 3 is not initiated yet. The experimental protocol for cell transplantation in SCID mice has been approved by the Institutional Animal Care and Use Committee (IACUC) of University of Illinois at Chicago (UIC). All research personnel associated with this project have successfully completed the Animal Care Committee (ACC) training for Animals and Research at UIC to the approved protocol. Experiments listed in Task 3 will be initiated soon.

KEY RESEARCH ACCOMPLISHMENTS:

- We found that hMSCs at the early passages (<passage 5) result in a significant inhibition on the development of morphine tolerance in neuronally differentiated SH-SY5Y cells in the co-culture, with a down-regulation of morphine-induced cAMP production as well as augmented expression of Rab5 and increased co-localization of Rab5 and MOR in the neuronally differentiated SH-SY5Ycells (23). A manuscript for publication is in preparation.

- We found that rMSCs at the early passages (passages 2-3) can produce significant analgesic and robust anti-tolerance effects in an animal pain model. A manuscript for publication is in preparation.

REPORTABLE OUTCOMES

Poster:

Yang HN, Sun JH, Wang F, and **Qu T***, Attenuation of chronic morphine-mediated cAMP upregulation in SH-SY5Y cells by mesenchymal stem cells, The 2nd International Conference and Exhibition on Addict ion Research & Therapy, Las Vegas, USA, July 22-24, 2013.

An abstract and a presentation are attached and support of DOD grant is acknowledged in the presentation.

Patents:

Qu T*, Cheng J, Sun J, and Yang HN, 2012/DG020, Human mesenchymal stem cells attenuate morphine tolerance (Office of Technology Management (OTM) of UIC).

Patent application with an abstract is attached and support of DOD grant is acknowledged.

CONCLUSION:

Our research is progressing well as planned in the SOW and will have a substantive impact on the final goals of this DOD grant. In addition, we are excited about the recent discovery that naïve hMSCs in the early passages (<passage 5) demonstrated an inhibition to the development of morphine tolerance and resulted in significant reduction to the morphine-induced cAMP production, augmented expression of Rab5, and increased co-localization of Rab5 and MOR in neuronal cells differentiated from SH-SY5Y cells (23). Consistent with these *in vitro* findings, the results of *in vivo* experiments in our collaboration studies with Dr. Cheng (Initiating PI) confirmed that spinal transplantation of rMSCs of early passages (passage 2-3) produced significant analgesic and robust anti-tolerance effects of in rat pain model. We are continuing to implement our research plans as outlined in our original research proposal by focusing to the SOW. We will compare the therapeutic value, analgesic effect, and anti-tolerance capacity of the early passaged naïve MSCs and the CLCs (reprogrammed MSCs) to expand our research and determine the best cell type for potential clinical application. We will also compare intravenous vs. intrathecal application of these cells to determine the best route of clinical practice. In this way, we hope to move much closer to treat chronic pain with this novel treatment to produce long-lasting significant analgesic and robust anti-tolerance effects.

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APPENDICES:

- 1 Abstract
- 1 Patent with abstract
- 1 Publication

AddictionTherapy-2013

OMICS Group Conferences
Accelerating Scientific Discovery

2nd International Conference and Exhibition on
Addiction Research & Therapy

July 22-24, 2013 Las Vegas, USA

Attenuation of chronic Morphine-mediated cAMP upregulation in SH-SY5Y cells by mesenchymal stem cells

Hongna Yang, Jinhua Sun, Feng Wang, and Tingyu Qu
University of Illinois at Chicago, USA

Abstract

The cAMP upregulation induced by chronic morphine is regarded as one of the molecular mechanisms leading to its tolerance and dependence. In the present work, we differentiated SH-SY5Y cells into neuron-like cells by retinoic acid (RA), pretreated these cells by morphine, the highly addictive drug, and tested their cAMP levels under different conditions, including co-culture with bone marrow-derived human mesenchymal stem cells (hMSCs) and human dermal fibroblast cells (hDFCs). We found that chronic treatment with 10μM morphine led to cAMP upregulation in these differentiated SH-SY5Y cells and the morphine mediated-cAMP upregulation was significantly attenuated by co-culturing with the hMSCs at early passages (P≤5), though this attenuation did not occur in co-cultures with the hMSCs at late passages (P>5) or hDFCs. In addition, hMSCs improved the mu-opioid receptor-mediated endocytosis in SH-SY5Y cells in preventing the development of morphine tolerance. In summary, early passaged hMSCs can successfully inhibit morphine induced cAMP upregulation in RA-differentiated SH-SY5Y cells by cell-cell contact and/or their released molecules, suggesting that hMSCs may serve as valuable therapeutics for treating morphine tolerance and dependence to minimize the risk of drug abuse and addiction. This research is supported by grants of DoD (PR100499P1) & the Boothroyd Foundation to T. QU.

Biography

Hongna Yang is a visiting student of Psychiatry of UIC, from Shandong University in China. Her focus is on the stem cell therapy (NSCs, MSCs) of neurodegenerative diseases and morphine tolerance.

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*Report of Research Discovery**

to the Office of Technology Management

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Primary Investigator Information

Name: Tingyu Qu Department: Psychiatry
Email: tqu@uic.edu, tqu@psych.uic.edu Phone: 5-1786
(please note that the Primary Investigator will be our main contact for all future correspondence)

Questions

1. Please provide a title and attach a full description of this discovery - manuscripts and grant proposals make excellent descriptions.

Human mesenchymal stem cells attenuate morphine tolerance (An abstract of the discovery is attached)

2. Identify all funding sources for this research, such as research sponsors (governmental agencies, industrial sponsors, foundations, private agencies or others). If none, please write "None".

A. Name of sponsor or agency: DoD and the Boothroyd Fnd

Grant or contract no. #489633 and #558140

B. Name of sponsor or agency:

Grant or contract no.

3. Please list key search terms associated with your discovery:

i) Pain management
ii) Autologous mesenchymal stem cells
iii) Morphine
iv) Anti-tolerance and drug abuse

4. What do you think is unique about your discovery?

Autologous cell therapy in Pain management with a potential for anti-tolerance and drug abuse

5. What commercial products do you think your discovery could be useful for?

Autologous analgesic and anti-tolerance cellular products

***Full disclosure of research discovery is required.**

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*Report of Research Discovery**

to the Office of Technology Management

6. Optional: If you are aware of markets for your discovery, please list.

7. Optional: If you know of any companies who may be interested in your discovery, please list.

8. Which stage of development applies to your discovery?

	Theoretical concept		Working prototype exists
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X	Experimental proof of concept exists		discovery currently used outside the lab

If applicable, please describe timeline for planned future work:

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When? 09/19/12

b. Nature of disclosure?

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Name and contact info of other contributors to the discovery, if any:	Jinhua Sun, jsun@psych.uic.edu Hongna Yang, hongna@uic.edu
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Human mesenchymal stem cells attenuate morphine-induced tolerance (Patent application)

Human mesenchymal stem cells (hMSCs) affect the inflammatory milieu and release endogenous analgesic molecules such as met-enkephalins. These findings have led researchers to consider hMSCs as a treatment for various diseased conditions including painful states. Morphine is one of the effective pain killers commonly used in today's clinics. We recently investigated whether hMSCs can attenuate the morphine tolerance in morphine pre-treated SH-SY5Y cells by a cell co-culture system.

SH-SY5Y cell were pre-treated by 10μM morphine for 24h and then co-cultured with different passages of hMSCs or their conditioned media for 36 hours. cAMP level, which is involved in opioid tolerance development as one of the cellular mechanisms, was examined in the tolerated SH-SY5Y cells with and without cell co-cultures by a fluorescent immunoassay. As expected, chronic morphine treatment produced an up-regulated level of cAMP in SH-SY5Y cells. hMSCs at early passage (≤passage 5) significantly inhibited the up-regulation of cAMP level in SHSY5Y cells at the ratio of 1:5 and 1:25 (hMSCs/SH-SY5Y cells), with a gradual decline when the ratio of MSCs/SH-SY5Y cells was further decreased in co-cultures. However, hMSCs at late passage (≥passage 11) and conditioned medium of hMSCs show no significant inhibitory effects on the up-regulation of cAMP level in SH-SY5Y cells, suggesting that the effects of hMSCs in attenuating morphine tolerance seem to be cell-cell contact and cell passage dependent. These results provide new information that autologous hMSCs may attenuate the development the development of morphine tolerance produced by morphine application in the management of chronic pain.

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Targeted Cell Reprogramming Produces Analgesic Chromaffin-Like Cells From Human Mesenchymal Stem Cells

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Transplantation of allogeneic adrenal chromaffin cells demonstrated the promise of favorable outcomes for pain relief in patients. However, there is a very limited availability of suitable human adrenal gland tissues, genetically well-matched donors in particular, to serve as grafts. Xenogeneic materials, such as porcine and bovine adrenal chromaffin cells, present problems; for instance, immune rejection and possible pathogenic contamination are potential issues. To overcome these challenges, we have tested the novel approach of cell reprogramming to reprogram human bone marrow (BM)-derived mesenchymal stem cells (hMSCs) using cellular extracts of porcine chromaffin cells. We produced a new type of cell, chromaffin-like cells, generated from the reprogrammed hMSCs, which displayed a significant increase in expression of human preproenkephalin (hPPE), a precursor for enkephalin opioid peptides, compared to the inherent expression of hPPE in naive hMSCs. The resultant chromaffin-like cells not only expressed the key molecular markers of adrenal chromaffin cells, such as tyrosine hydroxylase (TH) and methionine enkephalin (Met-enkephalin), but also secreted opioid peptide Met-enkephalin in culture. In addition, intrathecal injection of chromaffin-like cells in rats produced significant analgesic effects without using immunosuppressants. These results suggest that analgesic chromaffin-like cells can be produced from an individual's own tissue-derived stem cells by targeted cell reprogramming and also that these chromaffin-like cells may serve as potential autografts for chronic pain management.

Key words: Mesenchymal stem cells; Adrenal chromaffin cells; Cell reprogramming; Pain management; Autologous stem cells

INTRODUCTION

Transplantation of allogeneic adrenal chromaffin cells provides significant pain relief in patients with intractable cancer pain and in patients experiencing allodynia, a hallmark of neuropathic pain (1,11,13,25). There is often long-term pain relief without analgesic tolerance (1 year in humans) (24). Following adrenal chromaffin cell transplantation, application of exogenous opiates further alleviate pain without dosage escalation (25), that is, there was a stabilization of exogenous analgesic intake in these patients, strongly indicating that chromaffin cell grafts ameliorated the problem of opioid tolerance (12,13). These adrenal chromaffin cells release a "cocktail" of endogenous analgesic substances, including enkephalins, catecholamines, γ-amino butyric acid, indolalkylamines, and other neuropeptides (15,42). The analgesic effects of chromaffin cells can be partially reversed by intrathecal injection of either the opioid antagonist naloxone or the adrenergic antagonist phentolamine (9,32,33), suggesting that these analgesic effects are mediated by the opioids and catecholamines released by these cells. The antitolerance effects produced by the transplantation of adrenal chromaffin cells may be attributed to the synergistic action of the endogenous molecules released by these transplanted cells.

Although transplantation of allogeneic chromaffin cells demonstrates a viable modality of effective treatment for relieving pain and suffering in patients with the promise of ameliorating opioid tolerance, clinical practice has been hindered due to the limited availability of suitable human adrenal gland tissues. Mature chromaffin cells are postmitotic when they produce enkephalins and catecholamines. Expansion of these cells in culture is not possible. Xenogeneic materials, such as bovine and porcine chromaffin cells, have been extensively studied as potential alternative materials to human chromaffin cells. Transplantation of these xenogeneic cells into the spinal subarachnoid space produces antinociceptive effects on both Aδ and C fiber-mediated responses in rodents and in nonhuman primates (16,23,32–34,40) with a gradual

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decline in analgesic efficacy that can be prolonged by administration of immunosuppressants (23). Although chromaffin cells themselves are not very immunogeneic and highly purified chromaffin cells may minimize immunorejection (4,20), these experimental results suggest that, in transplants, xenogeneic chromaffin cells elicit immunological host rejection and that immunosuppressive therapy is necessary for enhancing long-term graft survival to extend the analgesic effect of these transplants. In addition, there has been concern regarding pathogen contamination by these xenogeneic materials, such as bovine spongiform encephalopathy in bovine chromaffin cell transplantation. Thus, the use of xenogeneic chromaffin cells presents serious problems.

The emerging approach of cell extract-based cell reprogramming developed by Håkelien and Collas et al. (3,5,8) represents a novel technology to furnish the phenotypic characteristics of the target cells to the reprogrammed cells for therapeutic purposes. We previously (41) reported that bone marrow (BM)-derived human mesenchymal stem cells (hMSCs) hold the inherent gene expression of preproenkephalin (PPE), a precursor for enkephalin opioid peptides, such as methionine- (Met-) and leucine- (Leu-) enkephalins, and in culture are able to release a low basal level of Met-enkephalin, a major neurotransmitter that plays an important role in analgesia by activating opioid receptors (15,33). To address the clinical needs for new and safe alternatives to adrenal chromaffin cells, recently, we further reprogrammed these hMSCs with nuclear and cytoplasmic extracts of porcine chromaffin cells and examined the phenotypic development and functional changes of the reprogrammed cells. RT-PCR assays revealed that the expression of human PPE (hPPE) in the reprogrammed hMSC population was greatly enhanced compared to that of naive hMSCs (unprogrammed). Immunoblot analysis confirmed that secretion of Met-enkephalin in serum-free culture of reprogrammed hMSCs was significantly augmented compared to secretions released by naive hMSCs for the same number of cells under identical conditions. Immunocytochemical examination demonstrated strong immunoreactivity in the reprogrammed hMSCs for Met-enkephalin and tyrosine hydroxylase (TH), specific markers for adrenal chromaffin cells. By targeted cell reprogramming, we have successfully developed a new type of cells from the reprogrammed hMSCs, which demonstrates the key phenotypic and functional features of adrenal chromaffin cells and thus termed these cells "chromaffin-like cells" (37,38). Transplantation of these chromaffin-like cells into the spinal intrathecal space of animals produced significant analgesic effects on both Aδ nociceptor- and C nociceptor-mediated responses in a rat thermal pain model. The analgesic effects lasted for 3 weeks without immunosuppression. Preliminary data from some of this work has been reported previously in abstracts (37,38).

MATERIALS AND METHODS

Chromaffin Cell Isolation, Purification, and Culture

Sixteen male and female porcine (1 year old) adrenal glands were obtained from a local slaughterhouse and immediately placed in ice-cold Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 5.6 mM glucose, 5 mM HEPES, pH 7.4; all components are from Sigma, St. Louis, MO, USA) supplemented with 100 UI/ml penicillin, 100 mg/ml streptomycin, and 0.125 ml/ml fungizone (PSF; Sigma-Aldrich) for transport. Preparation of isolation chromaffin cells was carried out using a method reported by us previously (39) with modifications. In brief, adrenal glands were manually perfused three times with warmed (37°C) Locke's buffer followed by digestion of connective tissue with 0.125% collagenase A (Boehringer Mannheim, Mannheim, Germany) in Locke's buffer for 3×5 min at 37°C. At the end of the digestion period, the chromaffin cells were isolated from the dissected medulla by mechanical dissociation. The harvested cells were filtered through a 70-μm nylon mesh (BD Falcon, Bedford, MA, USA) and then centrifuged at 150×*g* for 10 min in Locke's buffer. The resulting cells were purified on 39.47% Percoll gradients (Pharmacia Biotech, Uppsala, Sweden) by centrifugation at 22,500×*g* for 20 min. The portion of the gradients containing purified chromaffin cells was harvested by aspiration and washed three times by centrifugation at 150×*g* in Locke's buffer. Freshly isolated chromaffin cells were suspended and plated in 75-cm^2 culture flasks (Corning, Cambridge, MA, USA) containing Dulbecco's modified Eagle's medium/F-12 (DMEM/F12, 1:1; Gibco, Grand Island, NY, USA) supplemented with 10% (v/v) fetal bovine serum (FBS, Sigma) and antibiotics (PSF). These chromaffin cells were maintained at 37°C in a 5% CO_2 humidified incubation chamber (Fisher, Pittsburgh, PA, USA) fed by replacing culture media twice per week and used for experiments within 4 weeks after culturing because mature chromaffin cells are postmitotic and do not survive longer in cultured conditions.

hMSC Culture

In total, four samples of hMSCs (three males, ages 22, 25, and 32; one female, age 50), which are negative for surface markers associated with hematopoietic cells [e.g., cluster of differentiation 11b (CD11b), CD33, CD34 and CD133 antigens], were obtained from Cambrex (Walkersville, MD, USA). hMSCs were cultured and expanded using the protocol previously developed in our laboratory (30,39,41). In brief, hMSCs were plated in 75-cm^2 culture flasks at a concentration of 1×10^5 cells/cm^2 and cultured in 20 ml of serum-supplemented growth medium consisting of DMEM, an antibiotic–antimycotic mixture (1:100, Invitrogen, Carlsbad, CA, USA), and FBS (Stem Cell Technologies, Vancouver, BC, Canada), incubated at

37°C in a 5% CO_2 humidified incubation chamber, and fed by replacing half of the culture media twice a week. Cells were passaged by incubating with 0.05% trypsin–EDTA (Gibco) for 5 min at room temperature to gently release the cells from the surface of the culture flask after reaching about 80% confluency. Culture medium was added to stop trypsinization, and the cells were centrifuged at 350 × *g* for 5 min at room temperature, resuspended, and transferred into new culture flasks at a concentration of 1×10^5 cells/cm^2 for continuous culture and expansion to reach a sufficient number of cells. hMSCs that underwent less than 10 passages were used for the experiments.

Reprogramming hMSCs With the Extracts of Porcine Chromaffin Cells

The technology for cellular extract-based cell reprogramming, originally reported by Håkelien and Collas et al. (3,5,8), was applied by us with some modifications (37,38).

Preparation of Porcine Chromaffin Cell Extracts. Cultured porcine chromaffin cells were counted and washed twice in PBS (Sigma) and in cell lysis buffer (20 mM HEPES, pH 8.2, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and protease inhibitors; all from Sigma), sedimented at 400 × *g*, resuspended in 1 volume of cell lysis buffer, and incubated for 30 min on ice. Cell samples were then sonicated in 200 µl of aliquot on ice with a pulse sonicator (PowerGen 125, Fisher Scientific) in short pulses until all cells and nuclei were lysed and confirmed by microscopic observation. The lysate was centrifuged at 15,000 × *g* for 15 min at 4°C. The supernatant was aliquoted and stored in liquid nitrogen for later use. Before applying cell reprogramming, the protein concentrations of the cell extracts were determined (~30 mg/ml).

Permeabilization of hMSCs With SLO. hMSCs were resuspended from the culture, washed in Ca^{2+}- and Mg^{2+}-free PBS (Mediatech, Inc., Manassas, VA, USA), and centrifuged at 120 × *g* for 5 min at 4°C. The collected hMSCs were resuspended in aliquots of 1×10^5 hMSCs/100 µl of Ca^{2+}- and Mg^{2+}-free PBS in 1.5-ml tubes. Cell samples were permeabilized with streptolysin O (SLO; Sigma-Aldrich) at a final concentration of 200 ng/ml and incubated in an H_2O bath at 37°C for 50 min with occasional agitation to mix the cells. The cell samples were then placed on ice, diluted with 200 µl of cold PBS, and sedimented at 150 × *g* for 5 min at 4°C.

Reprogramming hMSC With the Extracts of Porcine Chromaffin Cells. The reversibly permeabilized hMSCs (1×10^5) were resuspended in 100 µl of the extracts of porcine chromaffin cells in a 1.5-ml tube containing an ATP-regenerating system (1 mM ATP, 100 µM GTP, and 1 mM of each NTP, 10 mM creatine phosphate, 25 µg/ml creatine kinase) (Sigma, St. Louis, MO, USA). Cell samples were incubated in an H_2O bath at 37°C for 1 h with occasional agitation. To reseal the membranes of hMSCs, 1 ml of DMEM containing 2 mM of $CaCl_2$ (Sigma) and antibiotics were added to the tube and incubated at 37°C for an additional 1 h. Finally, $CaCl_2$-containing DMEM was replaced by fresh DMEM with 10% FBS, and reprogrammed hMSCs were transferred to a 24-well plate (BD, Franklin Lakes, NJ, USA) at a concentration of 1×10^4 cells per well or into a 75-cm^2 culture flask at a concentration of 1×10^5 cells/cm^2 and incubated at 37°C in a 5% CO_2 humidified incubation chamber (Fisher). Twenty-four hours later, the floating cells were removed. The reprogrammed hMSCs grew well in culture and were expanded continuously, fed by replacing half of the culture media twice per week, and underwent regular cell passages when confluence was reached. Cells between passage 2 (1 week after cell reprogramming) and passage 4 (2 weeks after cell reprogramming) were used for experiments, and the viability of chromaffin-like cells was examined by trypan blue (Lonza, Walkersville, MD, USA), exclusion to be ≥95% at the time point before each experiment.

RT-PCR

The expression of hPPE genes in the population of chromaffin-like cells was analyzed by RT-PCR 1 week (passage 2) after cell reprogramming. Naive hMSCs with the same number of passages served as controls. RNAs from the cells were isolated using TRIzol reagents (Invitrogen) according to the manufacturer's protocol and treated with RNAase-free DNAase (Promega, Madison, WI, USA). The concentration of RNA was quantified by absorbance at 260 nm. RT-PCR was performed using a SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen) by means of specific primer sets for gene hPPE (forward: 5′-AC ATCAACTTCCTGGCTTGCGT-3′ and reverse: 5′-GCT CACTTCTTCCTCATTATCA-3′) and β-actin (forward: 5′-GACAGGATGCAGAAGGAGAT-3′ and reverse: 5′-TT GCTGATCCACATCTGCTG-3′). RT-PCR products were quantified using the Qgel 1D program (Stratagene, Cambridge, UK) and expressed as hPPE/β-actin (an internal control) ratio. In total, three independent experiments were performed for this experiment. Statistical analysis was performed using Student's *t* tests at a significance of $p < 0.05$.

Immunoblot

The production and secretion of Met-enkephalin opioid peptides in chromaffin-like cell culture was examined at 1 week (passage 2) following cell reprogramming using an established protocol of immunoblot assay as we performed previously (41). Cultured hMSCs with the same number of passages served as controls. In brief, cultured cells were resuspended from cell culture flasks and transferred onto a 12-well culture plate (BD) containing

serum-supplemented growth medium at a concentration of 1×10^5 cells/well. After recovering overnight, the serum-supplemented culture medium was replaced by serum-free culture medium. Twenty-four hours later, the medium was collected and purified with YM-30 microcon (Millipore Corp., Bedford, MA, USA). Purified medium samples (200 µl for each) obtained from chromaffin-like cells and naive hMSCs were applied to Hybond ECL nitrocellulose membranes (Amersham Life Science, Piscataway, NJ, USA) using the Slot Blot Hybridization Manifold (GENEMate, Kaysville, UT, USA). The membranes were blocked with 3% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) in PBS containing 0.5% Tween 20 (Sigma) (PBST) for 2 h at room temperature and then incubated with a specific Met-enkephalin rabbit antibody (1:600, ImmunoStar Inc., Hudson, WI, USA) overnight at 4°C. After washing three times with PBST, the membranes were incubated with anti-rabbit IgG peroxidase-linked species-specific whole donkey antibody (1:3,000, Amersham Biosciences, Piscataway, NJ, USA) for 90 min at room temperature. The membranes were washed with PBS, incubated with an ECL Plus detection reagent for 5 min, and then exposed to Hyperfilm ECL (Amersham Biosciences). The films were developed and scanned by a computer. The quantification of immunoblot band density was assessed by densitometric analysis using the NIH image program (ImageJ, NIH, Bethesda, MD, USA). Data were expressed as the mean ± SEM of four independent experiments. Student's *t* tests were performed to compare the levels of Met-enkephalin released by chromaffin-like cells with the levels released by naive hMSCs for the same number of the cells under the same condition of the serum-free cultures. Significance was set at $p < 0.05$.

Immunocytochemistry

Fluorescence immunocytochemical staining was performed on cultured chromaffin-like cells 2 weeks (passage 4) postcell reprogramming. Naive hMSCs served as controls. Cell samples in eight-well culture chambers (Thermo Scientific, Swedesboro, NJ, USA) were washed with PBS and fixed in 4% paraformaldehyde (Sigma) in PBS (pH 7.4) for 20 min at room temperature. To examine the proliferative potential of chromaffin-like cells, cell samples were incubated with 1 µM of bromodeoxyuridine (BrdU; Sigma) for 24 h and then treated with 2 N HCL (Fisher Scientific, Fair Lawn, NJ, USA) for 30 min. Following washing in PBS, cell samples were blocked in PBS buffer containing 0.1% Triton X-100 (Sigma-Aldrich) and 3% donkey serum (Jackson ImmunoResearch) for 30 min, followed by incubation with sheep anti-BrdU (1:500; Fitzgerald, Concord, MA), mouse anti-TH (1:300; Sigma-Aldrich), or rabbit anti-Met-enkephalin (1:600; ImmunoStar Inc., Hudson, WI, USA) antibodies overnight at 4°C. Then the cells were washed in PBS and incubated with their corresponding secondary antibodies, including rhodamine (TRITC)-conjugated donkey anti-sheep IgG (1:200; Jackson ImmunoResearch), fluorescein isothiocyanate (FITC)-conjugated donkey anti-mouse IgG, and rhodamine (TRITC)-conjugated donkey anti-rabbit IgG antibodies for 2 h at room temperature in the dark. Finally, the cells were washed with PBS, counterstained with DAPI (Vector Labs, Burlingame, CA, USA), and viewed by immunofluorescence microscopy (Zeiss, Jena, Germany). The number of cells fluorescently immunostained by a specific antibody and the number of cell nuclei stained by DAPI were counted in five randomly selected microscopic fields at 200× magnification. In total, three independent experiments were performed, and about 50–100 cells were counted for each experiment. The ratio of the cells fluorescently immunostained by specific antibodies to the total number of counted cells was recorded.

Pain Behavioral Tests

All animal experiments were performed in accordance with federal guidelines for proper animal care and an approved Institutional Animal Care and Use Committee protocol. In total, 12 adult male Sprague–Dawley (SD) rats (250–300 g body weight; Charles River Laboratories, Hollister, CA, USA) were used. Foot withdrawal latencies were measured before cell transplantation using high (Aδ nociceptor: 6.5°C/s) and low (C nociceptor: 0.9°C/s) rates of radiant heating on the dorsal surface of the feet according to the methods previously reported by us (16,17). The latencies from the onset of the stimulus to foot withdrawal responses were measured over 1 h at 10-min intervals. Chromaffin-like cells cultured 2 weeks (passage 4) after cell reprogramming were used. After the baseline for foot withdrawal responses was established, a single dose (1×10^5) of chromaffin-like cells in 20 µl of DMEM was injected intrathecally between the L4 and L5 lumbar vertebrae levels into the rats ($n = 6$) under anesthesia with pentobarbital (50 mg/kg, IP; Abbott Laboratories, North Chicago, IL, USA). We have observed previously that transplantation of this amount of chromaffin-like cells is appropriate to produce optimal analgesic responses in rats (37). Control rats ($n = 6$) received injections of the same amounts of naive hMSCs. Immunosuppressants were not used for cell transplantation. Foot withdrawal latencies were remeasured 1 week following cell transplantation and then weekly at 1 week intervals until latencies returned to baseline. Foot withdrawal latencies were expressed as the mean ± SEM. Data from baseline (before cell transplantation) and response latencies at different time points after cell transplantation were compared and analyzed using *t* tests followed by Bonferroni post hoc correction. Significance was set at $p < 0.05$.

RESULTS

hPPE Gene Expression of Chromaffin-Like Cells

One week after cell reprogramming with the cellular extracts of porcine chromaffin cells, RT-PCR was performed to examine the expression of hPPE. The molecular size of the RT-PCR product for the hPPE gene fragment was 425 bp. As expected, naive hMSCs demonstrated a low level of inherent hPPE gene expression. The chromaffin-like cells generated from the reprogrammed hMSCs showed a significantly enhanced expression profile for the gene hPPE compared to that of naive hMSCs (Fig. 1) ($p<0.01$), suggesting that cell reprogramming further increases the expression of hPPE genes in the population of reprogrammed hMSCs.

Met-Enkephalin Secretion of Chromaffin-Like Cells

In parallel to the time point examined for the expression of hPPE genes by RT-PCR (1 week postcell reprogramming), the serum-supplemented culture medium for chromaffin-like cell and naive hMSC cultures was replaced by a serum-free culture medium. Twenty-four hours later, the medium was collected and purified for Met-enkephalin detection by immunoblot assays. As shown in Figure 2, the level of Met-enkephalin released by chromaffin-like cells was significantly augmented compared to that released by naive hMSCs for the same number of cells (1×10^5 cells/well) in serum-free cultures ($p<0.01$). Although naive hMSCs were able to produce and release a low level of Met-enkephalin into the serum-free medium, augmented production and secretion of Met-enkephalin opioid peptides in chromaffin-like cells were consistently observed in each of the four independent experiments.

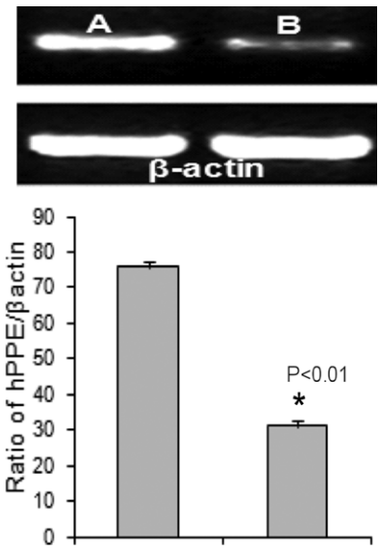

Figure 1. The expression of hPPE gene in the chromaffin-like cells was analyzed by RT-PCR at 1 week (passage 2) after cell reprogramming. Naive human mesenchymal stem cells (hMSCs) with the same number of passages served as controls. RT-PCR products (425 bp) were quantified using the Qgel 1D program (Stratagene, Cambridge, UK) and expressed as human preproenkephalin (hPPE)/β-actin (an internal control) ratio. In total, three independent experiments were performed. Statistical analysis showed that hPPE expression of chromaffin-like cells (A) is significantly upregulated compared to that of naive hMSCs (B) (*$p<0.01$, Student's *t* test).

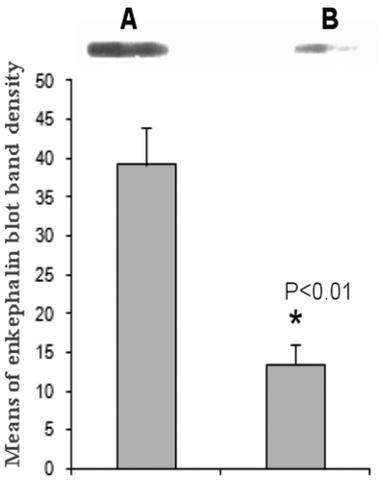

Figure 2. The secretion of Met-enkephalin opioid peptides of chromaffin-like cells (1×10^5 cells) in serum-free culture was examined by an immunoblot at 1 week (passage 2) after cell reprogramming. Naive hMSCs (1×10^5 cells) with the same number of passages served as controls. Twenty-four hours after culturing, the medium sample was collected and purified for immunoblot assay. The quantification of immunoblot band density was assessed by densitometric analysis using the NIH image program (ImageJ). Data were expressed as the mean ± SEM of four independent experiments. Statistical analysis showed that the Met-enkephalin secretion of chromaffin-like cells (A) is significantly augmented compared to that of naive hMSCs (B) for the same number of cells under the same culture condition (*$p<0.01$, Student's *t* test).

Immunocytochemical Examination of Chromaffin-Like Cells

Following cell reprogramming, morphological changes in hMSCs were observed within the first few days, that is, reprogrammed hMSCs became smaller and rounder. Five days later, the cells reverted to fibroblast-like shapes. These cells expanded in culture at a speed slightly slower (doubling time: about 84 h) in the first week and recovered to a normal dividing rate (doubling time: about 72 h) similar to that of naive hMSCs. Two weeks after cell reprogramming, immunocytochemical examination showed that most of the resultant chromaffin-like cells (≥90%) expressed a strong immunoreactivity for Met-enkephalin and TH (Fig. 3), specific cytoplasmic markers for adrenal

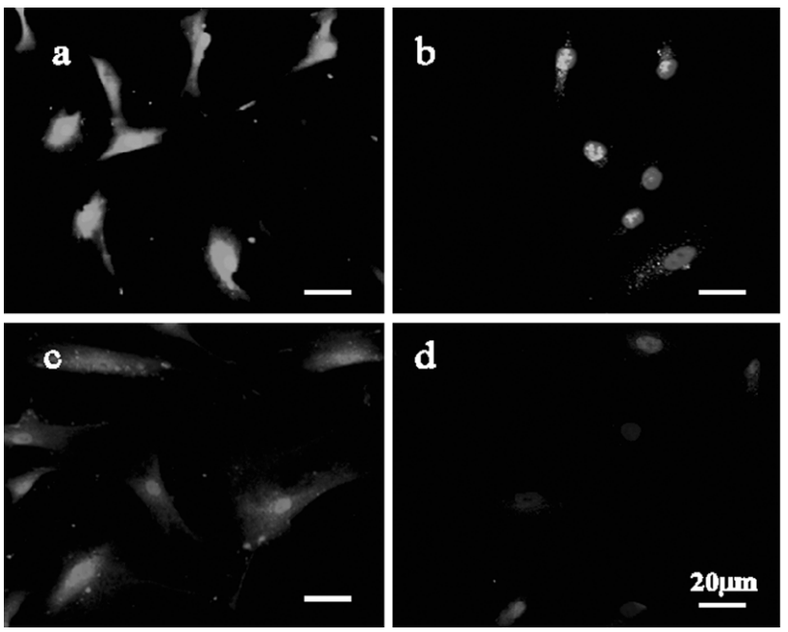

Figure 3. Fluorescence immunocytochemical staining was performed on cultured chromaffin-like cells 2 weeks postcell reprogramming (passage 4). Naive hMSCs with the same number of passages served as controls. Micrographs showed that chromaffin-like cells expressed strong immunoreactivity for tyrosine hydroxylase (a) and Met-enkephalin (c), while naive hMSCs expressed weak immunoreactivity for tyrosine hydroxylase (b) and Met-enkephalin (d). DAPI stained the nucleus of all cells. Consistent results were received in three independent experiments. Scale bar: 20 μm.

chromaffin cells. Interestingly, BrdU-positive staining was detected in a subpopulation of chromaffin-like cells (data not shown), suggesting that some chromaffin-like cells may have retained a similar proliferative capability as that of hMSCs and could be expandable in cell cultures. These chromaffin-like cells remained stable phenotypes and expanded in cultures with an average cell doubling time of about 72 h up to 1 month, undergoing about eight passages. We did not maintain the culture of the cells past the 1-month time point after cell reprogramming.

Analgesic Effects of Chromaffin-Like Cells

The analgesic effects of the chromaffin-like cells to a noxious thermal stimulus were investigated with the Aδ and C fiber-mediated foot withdrawal latency tests in rats. Transplantation of chromaffin-like cells into the subarachnoid space of rats produced remarkable analgesic effects on both Aδ and C fiber-mediated responses, which were evoked by a high and a low heating rate, respectively, and increased foot withdrawal latencies for 3 weeks in the absence of immunosuppression, with significant increases compared to the baselines of foot withdrawal latencies for 2 weeks (Fig. 4) ($p<0.01$). The analgesic effects of chromaffin-like cells were greater for C fiber- than for Aδ fiber-mediated responses. The maximum analgesic effects were observed in the first week following cell transplantation, and analgesic efficacy declined gradually with time. Control rats with naive hMSC transplantation showed similar response latencies to those measured previously in rats without cell transplantation (data not shown). No adverse effect was observed in the rats either with chromaffin-like cell or with naive hMSC transplantation.

DISCUSSION

Cellular extract-based cell reprogramming technology makes it possible to develop new types of cells by reprogramming one type of cells with extracts derived from a chosen targeted cell type to furnish reprogrammed cells with the phenotypic characteristics of the target cells (3,5,8). For example, incubation of a cell line, 293T fibroblasts, in the nuclear and cytoplasmic extracts of human T-cells resulted in the reprogrammed fibroblast cells taking on T-cell properties, expressing T-cell-specific surface molecules, and assembling the interleukin-2 receptor in response to T-cell receptor CD3 stimulation—a complex regulatory function (8). By using a similar approach, a related study demonstrated the induction of the pancreas-specific genes pancreatic and duodenal homeobox 1 (Pdx1) and insulin in rat primary fibroblasts treated with an extract of rat insulinoma cells (6). In the current studies, we reprogrammed hMSCs with nuclear and cytoplasmic extracts from porcine chromaffin cells, and the reprogrammed hMSCs demonstrated

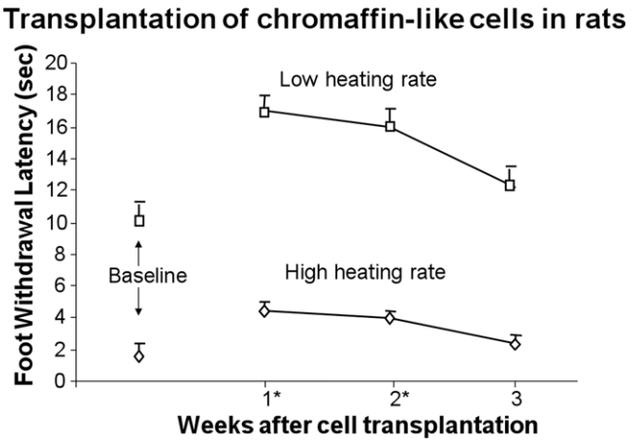

Figure 4. Effects of intrathecal transplantation of a single dose (1×10^5) of chromaffin-like cells on foot withdrawal latencies evoked by low (C nociceptor: 0.9°C/s, squares) and high (Aδ nociceptor: 6.5°C/s, diamonds) heating rates on the dorsal surface of the feet in rats ($n = 6$). Foot withdrawal latencies were measured before cell transplantation (baseline) and remeasured 1 week and then weekly at 1-week intervals following cell transplantation. Data from baseline and response latencies at different time points after cell transplantation were expressed as the mean ± SEM and compared using *t* tests. Follow-up analysis was performed by *Bonferroni post hoc tests.* Results showed that transplantation of chromaffin-like cells increased foot withdrawal latencies evoked by both high and low heating rates for at least 3 weeks, with significant increases compared to baseline for 2 weeks (*$p < 0.01$, *t* tests and *Bonferroni correction*).

phenotypic and functional characteristics similar to those of true chromaffin cells.

As previous studies have shown, hMSCs are a subset of self-renewing multipotent stem cells and are capable of differentiating into various mesenchymal cell lineages, including bone, cartilage, fat, tendon, and other connective tissues (18,26,28). Several studies have reported that hMSCs can also transdifferentiate into a diverse family of cell types unrelated to their phenotypic embryonic origin, including muscle and hepatocytes (14,19,27), as well as neural cells (2,30,35). Recent studies, including ours, have revealed that hMSCs display an inherent gene expression of hPPE and spontaneously release a low level of Met-enkephalin in culture (29,41). These cells are preferred candidates for our targeted cell reprogramming not only because of their latent capability to produce analgesic substances and plasticity for multitransdifferentiation but also because they are relatively easy to isolate from an individual's own tissues and are able to be expanded in culture with a regular doubling time as well as low levels of senescence during repeated passages. We choose porcine adrenal chromaffin cells as reprogramming materials because these cells share characteristics with human adrenal chromaffin cells in many respects, such as synthesizing and releasing opioid peptides and other pain-inhibitory compounds, including enkephalins and catecholamines (16,40). Also, compared with bovine chromaffin cells, porcine chromaffin cells are more potent in producing analgesia as transplants (16). In addition, porcine chromaffin cells are considered a safe source of cells that are readily available in large quantities—in fact, pigs have been cloned and are now being bred for whole-organ transplantation in humans (21,22).

Consistent with previous studies (29,41), our results demonstrate that naive hMSCs are able to express a low level of hPPE genes and to release a basal level of Met-enkephalins into a serum-free culture medium. However, chromaffin cell extract-based cell reprogramming significantly increases the expression of hPPE and the production and release of analgesic molecule Met-enkephalin, a neurotransmitter that plays a major role in analgesia by activating peripheral opioid receptors, in reprogrammed hMSCs (Figs. 1 and 2) ($p < 0.01$). As shown by our immunocytochemical examination, about 90% of the resultant chromaffin-like cells expressed strong Met-enkephalin and TH (Fig. 3), key markers of chromaffin cells. We did not examine the levels of catecholamines released by chromaffin-like cells in this study; however, TH is an enzyme controlling the rate-limiting step of catecholamine biosynthesis and is specifically found in the cytoplasmic matrix of cells containing catecholamines (31,36); positive immunoreactivity for TH may suggest that these chromaffin-like cells have the potential to produce catecholamines. More interestingly, BrdU immunoreactivity was detected in a subpopulation of chromaffin-like cells (30%), suggesting that these cells may have retained the proliferative properties of hMSCs and may make these cells even more valuable because generation of dividing

cells that are expandable in culture would provide a sufficient quantity of such cells for targeted use. In practice, the analgesic potential and secretion activity of chromaffin-like cells for enkephalins can be further manipulated in continuous cultures and thus become more powerful by using targeted gene transfection and/or cell fusion techniques, as demonstrated in our previous studies (39,41). As revealed by RT-PCR, immunocytochemistry, and immunoblot analyses in our studies, reprogrammed hMSCs demonstrated similar phenotypic and functional characteristics of chromaffin cells by targeted cell reprogramming. Although the mechanism for cell reprogramming is not fully understood, it is suggested that nuclear and cytoplasmic extracts may contain regulatory components that mediate alterations in the gene expression profile of the target genome and promote the nuclear importation of nuclear regulatory components (7,10). It is likely that chromaffin cell extract-based cell reprogramming switches hMSCs from a mesenchymal program to a chromaffin-like program.

We further investigated the analgesic effects of chromaffin-like cells in vivo by spinal transplantation of the cells into the subarachnoid space of the rats. As shown in Figure 4, transplantation of chromaffin-like cells produced remarkable analgesic effects and significantly increased the foot withdrawal latencies mediated by both high (Aδ nociceptor) and low (C nociceptor) heating rates (Fig. 4) ($p<0.01$). The analgesic effects of chromaffin-like cells lasted for 3 weeks in rats without immunosuppression. Although we did not make observations on the secretion of chromaffin-like cells past the 1-week time point of cell reprogramming in our in vitro studies, the analgesic effects of chromaffin-like cells demonstrated in living animals suggest that these cells can continuously release analgesic substances in vivo after transplantation. In addition, a more robust analgesic potential of chromaffin-like cell grafts compared to that of the hPPE-transfected NT2 (human neuron-committed teratocarcinoma) cell grafts as reported in our previous studies was found (17), that is, a low number of chromaffin-like cells (1×10^5) produced similar analgesia effects to that of a high number of hPPE-transfected NT2 cells (1×10^7) in the same animal models. The duration of the analgesic effects produced by chromaffin-like cells was similar to that of the porcine chromaffin cells reported previously (16), with a time-dependent gradual decline in analgesic efficacy. Although we did not perform histological examination for the grafted cell fate in this study, these results suggest that host immune responses to the transplanted xenogeneic cells may occur. As demonstrated by our previous study (23), only few xenogeneic cells could be detected in the transplanted animals without immunosuppression, suggesting that administration of immunosuppressants is necessary to maintain long-term survival and long-lasting analgesic effects of xenogeneic cell grafts. Further studies are warranted to determine the survival rate, phenotypic stability, and secretion activity of chromaffin-like cells in vivo by using autologous cell transplantation in related animal pain models.

It is common knowledge that use of immunosuppressants can have severe side effects, including tumor formation, and should be avoided when possible. Transplantation of cells derived from individuals' autologous tissues into the same individuals would be safe and immunocompatible compared to xeno- and allotransplants. In practice, hMSCs can be derived from a patient's own tissues and not only could be prepared to become isogeneic through this cell reprogramming approach but also could avoid immunological rejection through autologous cell transplantation. Thus, autologous chromaffin-like cells could be the most desirable alternative to adrenal chromaffin cells for potential therapeutic purposes. Robust and long-lasting analgesic effects of autologous chromaffin-like cells are expected because these cells would be spared from immune responses, thereby improving the therapeutic efficacy of the transplanted cells.

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